

FINE STRUCTURE AND SPINE FORMATION IN *TREUBARIA* (CHLOROCOCCALES) : A SYNTHESIS AND NEW RESULTS

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ABSTRACT. — Based on light, TEM and SEM investigations of field and culture-grown materials, the common features of the four species of *Treubaria* which constitute this genus (sensu REYMOND, 1980) are described and discussed : centrioles, phycoplast, pyrenoidal cytoplasmic invaginations, delays in furrowing, naked spores, changes of cell wall and polymorphism, the net of fibrils constituting the cell wall and the empty spines. The elaboration of spines (which also involves the cell wall) with the participation of Golgi vesicles and a new structure called «collar» situated like a ring at the base of the young processes is emphasized. Taxonomical remarks and ultrastructural comparisons are made concerning the relationship of *Treubaria* with other genera of the family Treubariaceae (*Desmatriactum*, *Pachycladella* and *Echinosphaerella*), other chlorococcalean spine bearing organisms and *Dicranochaete* (Tetrasporales).

KEY WORDS : Ultrastructure, taxonomy, cell wall, spine formation, collar, phycoplast, Green alga, Chlorococcales, Treubariaceae, *Treubaria*.

INTRODUCTION

The genus *Treubaria* Bernard (1908) includes four freshwater planktonic and unicellular green algal species. The main light microscopical feature is a lobate, polyedrial or spherical cell body surrounded with at least three translucent and colorless spines, processes or cones. Reproduction is by formation of non motile spores (naked with contractile vacuole). Quadriflagellate zoospores have also been observed (REYMOND, 1979, 1980).

The prominent works dealing with this genus began with ARCHER (1872) who observed *Treubaria* for the first time and described it as *Tetrapedia setigera* (= *Treubaria setigera* (Archer) G.M. Smith, 1933) as a blue green alga.

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BERNARD (1908) and G.M. SMITH (1933) provided additional information on *Treubaria*, followed by the revision by KORSIKOV (1953). FOTT & KOVACIK (1975), REYMOND (1980) in the short summary of his thesis work, and KOMAREK & FOTT (in HUBER-PESTALOZZI, 1983) in their huge revision of Chlorococcales complete this list of selected papers dealing mostly with light microscopy results and taxonomy.

The electron microscopy publications started with the short papers by REYMOND & JALANTI (1976a, 1976b). They were followed by papers by REYMOND (1979, 1980) and REYMOND & CRONBERG (1981). This present work presents more ultrastructural details than the earlier papers and emphasizes new results concerning the very puzzling spine formation in young spores, a phenomenon rarely described in phycological publications.

Ultrastructural studies also concern taxonomy. Taxonomy in *Treubaria* is not yet clear and depending on from which author the information comes, this genus can include a couple or more species. This present work is taxonomically based on the previous work by REYMOND (1980) in which only four species compose the genus : 1) *Treubaria setigera* (Archer) G.M. Smith (1933); 2) *T. triappendiculata* Bernard (1908); 3) *T. schmidlei* (Schröder) Fott & Kovacik (1975); 4) *T. crassispina* G.M. Smith (1926). Each of these four species have, when observed with light and electron microscopes, been found to be more or less suitable for specific kinds of investigations. Although the ultrastructure of each species of *Treubaria* can differ, the present work is not a comparative study, but rather a description, of the common features and a comparison with other genera, e. g., *Desmatriactum* West & West (1902) or *Pachycladella* (G.M. Smith) Silva (1970).

Some of the following results are a short part of the unpublished complete thesis work of the author, which deals with taxonomy, nomenclature, bibliography, iconography and biology, and is entitled «Contribution à l'étude de *Coelastrum* et *Treubaria*» (1-780 pp.) and can be consulted at the library of the «Musée botanique» in Lausanne, Switzerland.

MATERIALS AND METHODS

Treubaria setigera, *T. triappendiculata*, *T. schmidlei* and *T. crassispina* were used for this study. Observations with light microscope were made with field and culture-grown materials, whereas observations with Transmission Electron Microscope (TEM) and Scanning Electron Microscope (SEM) were made nearly only with culture-grown cells. Contrary to other species, *T. crassispina* could not be grown in culture and all of the specimens for TEM came from an old collection. A list including the origin of field and culture-grown materials was previously reported by REYMOND (1980). These cultures of *Treubaria* now are available from the algae collection, at the University of Göttingen, West Germany. They are incorporated (SCHLÖSSER, 1984) under the numbers : 36.83

T. schmidlei (strain Reymond 75-96); 37.83 *T. setigera* (strain Reymond 76-98); 38.83 *T. triappendiculata* (strain Reymond 72-69). Cultures were maintained in Bold's Basal Medium (BOLD, 1942) or in Pocock's Medium (POCOCK, 1960) at 20°C on a 16:8 L:D cycle. The culture were fixed for SEM following the method previously given by REYMOND & JALANTI (1976b) and were fixed for TEM with glutaraldehyde and osmium following recommendations of PICKETT-HEAPS et al. (1978). Samples were dehydrated in a graded acetone serie and embedded in Spurr's resin between two microscope slides (REYMOND & PICKETT-HEAPS, 1983). Sections were stained with uranyl acetate and lead citrate. The specific staining of polysaccharides on ultrathin sections was made on gold grids, with «periodic acid-thiocarbohydrazide-silver proteinate» technic described by THIÉRY (1967) and THIÉRY & RAMBOURG (1974). The control over the reactions was achieved by use of H₂O₂ instead of periodic acid as suggested by THIÉRY & RAMBOURG (1974). Processing of materials by freeze-etching technics (BULLIVANT, 1973) was achieved at the Nestle Laboratory in La Tour de Peilz, Switzerland. SEM pictures were taken at the «centre de Microscopie électronique de l'Université de Lausanne» with a JEOL 35. The TEM pictures were produced with Zeiss EM 10 and Philips (200 and 300) microscopes at the University of Lausanne, Geneva and Colorado (Boulder), and at the «Centre de Recherches agronomiques» in Changins, Switzerland.

OBSERVATIONS

General morphology of vegetative and dividing cells

The young vegetative cells have one laterally situated nucleus (Fig. 1) whereas the older cells contains two (Fig. 2), four or more central nuclei which can sometimes contain a ring shaped nucleolus (Fig. 3). During preprophase a pair of centrioles surrounded by many microtubules can be observed (Fig. 4). In spite of observations of numerous cells at different stages, mitosis could not be studied in detail. Post mitotic nuclei lie very close to each other and rudimentary centrioles appear in invaginations between them (Fig. 2, 3). From this region, numerous microtubules extend between the nuclei and form the phycoplast (Fig. 3) where furrowing later takes place. The phycoplast can be easily observed in many cells, the furrowing, on the contrary is infrequently observed due to its brief presence before sporulation. Formed spores are very compacted and do not possess any cell wall. During sporulation, the daughter cells rapidly increase their volume and escape through the disorganized mother cell wall (Fig. 5). The pressure in the naked spores is regulated through a contractile vacuole.

In many cases we observed that the furrowing (and consequently the sporulation) can be very late after the formation of two or four nuclei. In this case, the cells can present the following morphological changes: they can continue to multiply their nuclei by successive mitosis; their volume can increase notably; they can get rid of their three- or four-spined cell wall and form a new one with

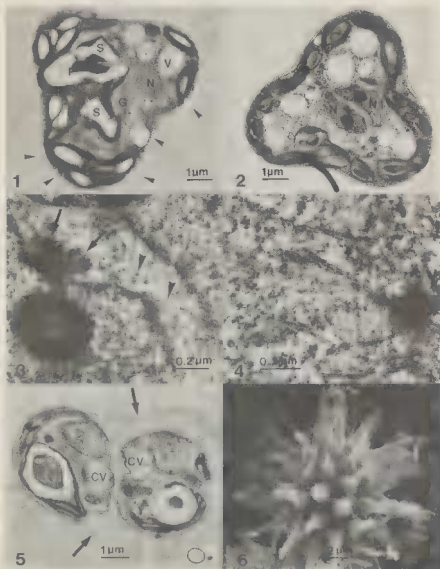


Fig. 1-4 : *Treubaria setigera*. — Fig. 1 : Cell with one lateral nucleus (N), walled chloroplast (Cl) with cytoplasmic invaginations in the pyrenoid (P). Golgi (G), starch (S), vacuoles (V). The cell wall and the spines are hardly visible (arrowheads). — Fig. 2 : Cell with two centrally opposed nuclei (N), with ring-shaped nucleoli. — Fig. 3 : Detail of Fig. 2. Between the two nuclei there is a phycoplast composed of rudimentary-shaped centrioles (arrows) and a plate of longitudinal and cross sectioned microtubules (arrow-

more spines; and a lobate cell can become polyedrial or spherical. Due to one or more of these changes, polymorphism is frequently observed amongst cells arising from the same unialgal culture.

Other ultrastructural features have been investigated in the cells of *Treubaria*. The outer membrane of the porous nucleus is always continuous with the endoplasmic reticulum. A transfer of vesicles between these three membranes can usually be observed (REYMOND, 1980). A significant part of the vegetative adult cells is filled with a cup-shaped parietal chloroplast (Fig. 1, 2). Stacks of long parallel thylakoids enclose several starch grains and the prominent starch cap of the pyrenoid (Fig. 1). The pyrenoid matrix is penetrated by a conspicuous network of cytoplasmic invaginations (Fig. 1, 5). In most cases these networks have no typical defined morphology but on rare occasions take a spherical shape (Fig. 7). The thylakoids never penetrate the starch cap of the matrix of the pyrenoid. The cytoplasm of the cells is filled with vacuoles (Fig. 1) which are no longer contractile when the cell wall is formed. However when a cell sheds its cell wall, a contractile vacuole immediately appears at its surface. The vacuole stops functioning when the new cell wall and the spines are completed.

The cell covering : morphology in vegetative cells and young spores

In vegetative cells, the protoplast is enveloped by a dense network of fibrils permeated with amorphous materials (Fig. 8, 9). These fibrils, alone or associated in thin bundles, constitute the frame of both cell wall and spines. The fibrils which constitute the cell wall seem to form a randomly ordered network (Fig. 10, 11). In fact SEM micrographs show that the fibrils (or at least many of them) radiate from the bases of the spines and surround the protoplast. Fibrils radiating from the base of one spine interdigitate with fibrils from the closest spines (Fig. 8, 9). Under these conditions, the directions of the fibrils and the complexity of the network that they form depend on the specific place at the surface of the cell and the number of spines (Fig. 12, 13). The thickness of the cell wall depends on the number of spines, the culture conditions and the species. The cell wall is very thick in *Treubaria crassispina* and *T. schmidlei* and is generally much thinner in *T. triappendiculata* and *T. setigera*.

The fibrils which constitute the frame of the spines are parallel (Fig. 14). They run along a very slightly helical trail from the top to the base of the spine where they form the cell wall (Fig. 8, 9). Except for the presence of some

heads). — Fig. 4 : Preprophase centriole surrounded by an important net of microtubules. — Fig. 5 : *Treubaria triappendiculata*. Daughter cells during their release from the mother cell wall (arrows). The new cell wall and the spines are not yet formed. Note the contractile vacuoles (CV). — Fig. 6 : *Treubaria schmidlei*. Cell with abnormally numerous spines. Such a cell can be observed both from field and culture-grown material. Taxonomical implications of this abnormality is explained in the text.



Fig. 7 : *Treubaria setigera*. — Section through pyrenoid (P) showing a spherical cytoplasmic invagination. — Fig. 8, 9 : *Treubaria schmidlei*. Fig. 8 : Slightly disrupted empty mother cell wall showing its intricate network of interdigitated fibrils radiating from the bases of the spines. The amorphous material gives a smooth appearance to the spines. — Fig. 9 : Detail of interdigitated fibrils radiating from the bases of three adjacent spines.

electron dense material forming a web between the fibrils constituting the frame, the inner part of the spines is free of cytoplasm and EM stained structures (Fig. 15).



Fig. 10 : *Treubaria schmidlei*. Freeze-etching showing the intricate network of fibrils which constitute the cell wall. Several levels and directions of fibrils can be observed. The complexity of the network depends on the number of spines from which fibrils radiate and interdigitate (Fig. 9). — Fig. 11 : *Treubaria triappendiculata*. Tangential section of the cell wall (CW) across a spine (S). — Fig. 12 : *Treubaria setigera*. Tangential section of two lobes of a trilobate cell (Fig. 1, 2) showing several layers of fibrils with various directions. Sections of spines are shown with arrows. — Fig. 13 : *Treubaria triappendiculata*. Cross section of a disrupted empty mother cell wall. The fibrils are — in longitudinal (arrows) and cross section (arrowhead).

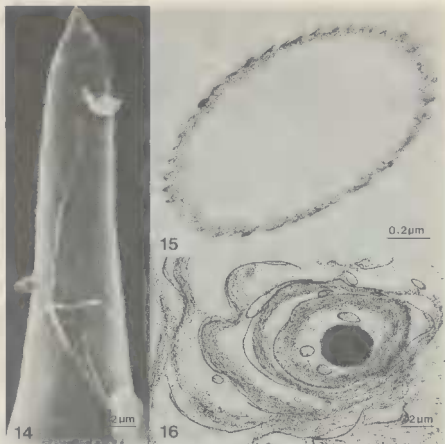


Fig. 14 : *Treubaria schmidlei*. The frame of the spine is made of parallel ordered fibrils.
 — Fig. 15 : *Treubaria setigera*. Slanting section through a spine. — Fig. 16 : *Treubaria triappendiculata* which has been grown on agar plate. Repeated changes of cell wall are frequent in *Treubaria*. This is especially obvious on agar, where nearly all the cell walls remain in the same place.

During sporulation and changes of cell wall, the latter can open in two ways. In some cases we observed a sliding apart of each spine accompanied by its basal radiating fibrils; in other cases the cell wall expanded. In the latter case the interdigitated fibrils slide slightly apart but stay together (Fig. 8, 9) when the spores escape through a slit made between the bases of the processes. The latter case which can be found in each species of *Treubaria* (with perhaps the exception of *T. crassispina*) was considered a characteristic feature of *Echinospaerella* (one species : *E. limnetica* G.M. Smith, 1920) by some phycologists.

In the description of cell division, we said that a cell can change its volume, shape, cell wall and the number of spines. These changes described by REYMOND & JALANTI (1976b) and REYMOND (1980) are very obvious in sections of agar-culture-grown cells (Fig. 16). Their taxonomic importance will be discussed later. For legal and technical reasons living material could not be imported from Brazil and the life cycle of *Treubaria crassispina* (which was collected in this country) could not be observed in detail. However changes of cell wall and shape could take place in this species, e. g., the multispined *Echinospaerella limnetica* shown by UHERKOVICH (1976) is certainly a *Treubaria crassispina* after one or more changes of cell wall.

Formation of spines

This part of the life cycle has been described with light microscopy by REYMOND (1979, 1980) and will be summarized for a better understanding of the TEM observations.

The spine and cell wall formation can easily be observed with phase-contrast microscopy and *Treubaria schmidlei* is a very suitable material. The formation starts immediately after the liberation of the spores (or just after the release of a previous cell wall). Asymmetrically placed, short, thin and dark processes can be observed at the surface of the naked cell, where a contractile vacuole is very active. These tiny processes are the future spines. They at first have an homogenous dark contrast then they slowly elongate and form an apical light contrast part and a basal dark one. At this step the spine is still very thin. The darker part contains cytoplasm and shortens while the lighter part continues to elongate to the maximum length of the future spine. During and shortly after the elongation, the spine thickens especially at its base and the darker basal part disappears completely. The mature spine is now totally translucent. The cytoplasm which had formerly invaded the young processes is now fully retracted into the spherical protoplast. The contractile vacuole is no longer active and the spines are at their full size and their final symmetrical positions around the protoplast. The cell wall is simultaneously formed with the growing spines. About fifteen minutes are needed for a spine to reach its full length and width, from its initial appearance. During their development, the spines seem to glide from their primary asymmetrical location to their final symmetrical position around the growing protoplast. Balances of tensions due to interdigitations of fibrils radiating from each forming spine could be the origin of these surprising movements. Even though very short immature spines are always asymmetrically placed at the surface of the protoplasts, rare observations indicate that there is an harmony, if one takes in consideration the pattern that they form inside the mother cell when the spores are not yet fully liberated (Fig. 17).

TEM investigations have been completed on half-developed spines of the four species of *Treubaria*, wherein nearly the same observations could be made. The young spores do not possess a real cell wall (Fig. 18, 19). The cytochemical test for polysaccharides (THIERY, 1967) reveals only a thin contrasted line

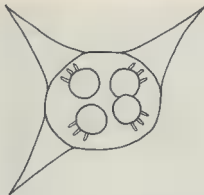


Fig. 17 : A schematic presentation of a rare observation made during a sporulation in *Treubaria schmidlei*. Even though the twelve short growing spines are asymmetrically positioned on each cell, they do show a symmetry together.

around the cytoplasm (Fig. 20 and controls, Fig. 21, 22) which certainly represents the young forming cell wall or even mucopolysaccharides. As revealed with phase-contrast, cytoplasm is found in the basal part of the growing spines and its surface takes a concave morphology in front of the distal empty part of the spine (Fig. 18-20, 23). A very peculiar structure, the «collar», is found at the bases of the young growing spines (Fig. 18-21, 24). The collar is a ring which surrounds the cytoplasm at the base of the spine. It is situated underneath the forming cell wall and it is not clear what its relationship with the plasmalemma is. It could be a thickening of the plasmalemma; however, its homogeneous matrix seldom shows contrasted sub-structure (Fig. 24). The diameter of the collar expands with the diameter of the base of the spine. The collar is never found in fully developed spines. The cytoplasm which can be found in the spines is filled with numerous and diverse vesicles (Fig. 18, 19). Most of them are generally rounded (Fig. 18, 19, 22) and their content shows a very positive reaction to the test for polysaccharides (Fig. 20 and controls, Fig. 21, 22). These vesicles seem to bring their contents to the surface of the cytoplasm through the forming spines (Fig. 20) and they seem to originate from the Golgi (Fig. 20). Other types of vesicles, of other size or other shape, with nearly only peripheral staining (for polysaccharides) can be found in the cytoplasm of forming spines. Their significances is not yet clear. Lipid vesicles can also be found around (Fig. 20) or sometimes in the forming spines. Their positive reaction (to the test for polysaccharides) on their surface has already been mentioned by THIÉRY (1967). The fibrils which form the frame of the young growing spines are not very numerous or densely stained (Fig. 23, 24) (in Fig. 18, they are negatively stained). Their origin cannot be elucidated easily but we think that they could be secreted, either at the level of the concave cytoplasm

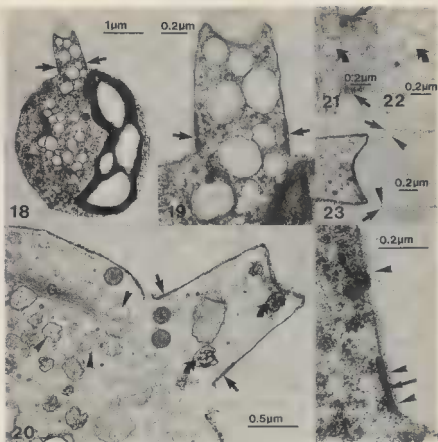


Fig. 18-23 : *Treubaria setigera*. — Fig. 18 : Section through a young cell and one of its forming spines. Cytoplasm with numerous vesicles is partly filling the forming spine. A collar (arrows) surrounds the base of the spine. The top of the spine is hardly visible and negatively stained. — Fig. 19 : Detail of fig. 18. : Note the concave surface of the cytoplasm and the presence of numerous vesicles filled with slightly stained materials. The collar is shown by arrows. — Fig. 20 : Detail of a section through a young cell and one of its forming spines. The polysaccharides are stained (with silver proteinate). The cell border and some vesicles (curved arrows) of the forming spines are well stained when several densities of contrast can be observed in the vesicles (arrowheads) around the Golgi (G). These vesicles are involved in the cell wall and the spines formation. Note the presence of lipidic vesicles (L) with stained periphery. The collar is shown by arrows. — Fig. 21-22 : Control section through a forming spine and vesicles (curved arrows). During silver proteinate staining procedure these sections have been treated with H_2O_2 instead of periodic acid. Polysaccharides can no longer react with silver proteinate. The cell border and the vesicles contents are not densely stained. The collar is shown by arrows (cf. with Fig. 20). — Fig. 23 : A forming spine stained with silver proteinate. The fibrils which form the spine seem to originate from the side (arrows) and from the top of the concave cytoplasm (arrowheads). — Fig. 24 : *Treubaria schmidlei*. Note the heterogeneity of the collar (arrow) and the fibrils which only can be observed along side and over it (arrowheads). The role of the collar in the spines formation is not yet clear.

(Fig. 23) or at the level of the collar (Fig. 24). The paucity of material at the right stage has hindered us from obtaining additional interesting results on this problem, with other methods (e. g., freeze-fracturing).

DISCUSSION

These investigations of the ultrastructure of *Treubaria* have shown that some of its morphological features are common to other green algae or Chlorococcales. Others may be specific for *Treubaria*.

The information concerning mitosis is incomplete. However we have found that the mitosis seems similar to that of Chlorococcales where, as in *Treubaria*, a phycoplast was observed, e. g., *Kirchneriella* (PICKETT-HEAPS, 1970), *Tetraedron* (PICKETT-HEAPS, 1972), *Sorastrum* (MARCHANT, 1974), *Scenedesmus* (PICKETT-HEAPS & STAEHELIN, 1975), *Coelastrum* (MARCHANT, 1977), *Nautococcus* (DEASON & O'KELLY, 1979). The presence of a perinuclear envelope of endoplasmic reticulum during mitosis is still hypothetical. As in *Kirchneriella* (PICKETT-HEAPS, 1970), pairs of centrioles with conventional morphology seem to appear only at prophase. Phycoplast centrioles look very rudimentary.

In contrast to other algae wherein phycoplast formation is followed closely by furrowing, cleavage and spore release, *Treubaria* can sometimes use another cycle in which the long delay in furrowing has some consequence. Taxonomically the result is that cells which undergo these changes in morphology were considered similar to *Echinosphaerella* G.M. Smith (1920). We consider that *E. limnetica* is in fact *Treubaria schmidlei* (Schröder) Fott & Kovacik (1975). This opinion is supported by many experiments with a large amount of field and culture grown material as well as by complete bibliographical research concerning *Treubaria* and the other genera of the family *Treubariaceae* (c. f. the last paragraph of Introduction). A few specific remarks can be made concerning *Treubaria crassispina*. We observed this species, which has never been culture-grown, only in fixed field samples wherein we never found any *Echinosphaerella*-like cell. However, *Echinosphaerella limnetica* drawn by UHERKOVICH (1976) from Amazonian material could represent *Treubaria crassispina* as mentioned in the Observations.

The ultrastructure of *Treubaria* reveals another characteristic of this genus : the intraplastidic pyrenoid is always penetrated by cytoplasmic invaginations. Although this characteristic also can be observed in many other algae, it was never found in the other members of the family *Treubariaceae* observed with TEM : *Pachycladella* (REYMOND & HEGEWALD, in preparation) and *Desmatractum* (REYMOND & KOUWETS, 1984). On one occasion, the cytoplasmic invagination in *Treubaria setigera* was found to be spherical, as in the Tetrasporales *Dicranochaete* observed by VAN DE WIEL & REYMOND (1983). However, if this detail is worth mentioning at all, it is in any case too early to

base taxonomical relationships on this feature alone.

Based on a light microscopic level test made with sulphuric acid, KORSIKOV (1953) supposed that the cell wall of *Treubaria* was composed of cellulose. However the real specificity of this test for cellulose is questionable. Our TEM investigations reveal that the cell wall, the spines and some specific vesicles show a positive reaction to the polysaccharide test of THIÉRY (1967). The true nature of the cell wall is very peculiar and does not show any layer as well differentiated as those of most Chlorococcales, e. g. *Nautococcus* (DEASON & SCHNEPF, 1977) or *Chlorella* (DEMPSEY et al., 1980). Some similarities with the fibrillar cell wall of *Oocystis* (SACHS et al., 1976) can be observed. However, the network of fibrils in *Treubaria* seems much more complicated and intricate, due to the presence of numerous spines. The origin of the fibrils in the cell wall of *Treubaria* is not yet clear. We suppose that the vesicles observed in the forming spines and the collar are involved in this procedure. This hypothesis, based on the fibrils' orientations and interdigitations does not exclude that other fibrils also could be secreted later elsewhere around protoplast. There are no similarities between the spines and the cell wall of *Treubaria* and the other members of the family Treubariaceae investigated with TEM, e. g. in *Desmatractum*, all the species possess a cell wall which is well differentiated from the spines, and the texture of the spines is not at all like that of *Treubaria* (REYMOND & KOUWETS, 1984); identical remarks can be made about *Pachycladella*, in which the cell wall is strong and spines tubular and weak (REYMOND & HEGEWALD, in preparation). Furthermore we think that no similarities to *Treubaria* can be found in the bristles or spikes shown in *Acanthosphaera*, *Micractinium*, *Pediastrum*, *Polyedriopsis*, *Scenedesmus*, *Siderocystopsis*, *Golenkinia* and *Echinospaeridium* (all Chlorococcales) by SCHNEPF et al. (1980) and HEGEWALD & SCHNEPF (1984). The formation of spines or appendages is rarely investigated in algae, and when it is, the investigations never concern empty ones like those of *Treubaria*. To our knowledge, vesicles and collars, like those in *Treubaria* have never been found in the forming spines of other algae. This does not preclude that they could be found later in other species which bear empty conical spikes. In contrast to *Treubaria*, numerous microtubules were found in the forming processes of *Sorastrum* and *Pediastrum* wherein no retraction of cytoplasm can be observed (MARCHANT, 1974, 1979). It should also be mentioned as an historical note that BOURRELLY (1951) first observed cytoplasm in the growing spines of *Treubaria*. Unfortunately no other data concerning the life cycle of this alga were known and his accurate drawing remained without correct interpretation for a long time.

In conclusion, *Treubaria* seems to be notably different from other genera of Chlorococcales, and also from the other genera of the family Treubariaceae. According to all the authors who studied this family, it is an artificial assemblage.

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